Current Biology 19, 758–763, May 12, 2009 ©2009 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2009.03.018

Report

ICIS and Aurora B Coregulate the Microtubule Depolymerase Kif2a

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Summary

Kinesins in the mitotic spindle play major roles in determining spindle shape, size, and bipolarity, although specific regulation of these kinesins at distinct locations on the spindle is poorly understood. So that the forces that are required for spindle bipolarity are balanced, microtubule-depolymerizing kinesins are tightly regulated. Aurora B kinase phosphorylates the neck regions of the kinesin-13 family microtubule depolymerases Kif2a and mitotic centromere-associated kinesin (MCAK) and inhibits their depolymerase activities. How they are reactivated and how this is controlled independently on different kinetochore fibers is unknown. We show that inner centromere Kin-I stimulator (ICIS), which stimulates the related depolymerase MCAK, can reactivate Kif2a after Aurora B inhibition. When antibodies that block the ability of ICIS to activate Kif2a are injected into cells, monopolar spindles are generated. This phenotype is rescued by coinjection of anti-Nuf2 antibodies. We have performed a structure-function analysis of the ICIS protein and find that the N terminus of ICIS binds Aurora B and its regulators INCENP and TD60, whereas a central region binds MCAK, Kif2a, and microtubules, suggesting a scaffold function for ICIS. These data argue that ICIS and the chromosomal passenger complex (CPC) regulate Kif2a depolymerase activity.

Results and Discussion

The C Terminus of ICIS Reverses Aurora B Inhibition of Kif2a In Vitro

The microtubule depolymerase activities of Kif2a and mitotic centromere-associated kinesin (MCAK) are inhibited through phosphorylation by Aurora kinases [1–3]. On MCAK, this phosphorylation event has been mapped to the neck region, which lies proximal to the kinesin domain. Not only does Aurora regulate MCAK activity, but it also regulates its localization on the spindle [1, 3, 4]. Surprisingly, phosphorylated MCAK is often found at locations where MCAK activity is required [5, 6]. Therefore, there must be tight spatial regulation of the activity of these proteins in their different subcellular locations, as well as mechanisms to reactivate depolymerase activity after Aurora phosphorylation.

We adapted a system to measure Kif2a microtubule depolymerase activity in vitro [1]. Rhodamine-labeled taxol-stabilized microtubules were incubated with recombinant Kif2a aa 118–530, which is the minimal domain required for depolymerase activity (Kif2a minimal domain). Although this protein construct was cloned from the human sequence, it is 93% identical to the *Xenopus* sequence (Figure S1 available online). Titration of Kif2a into the reaction shows concentration-dependent depolymerase activity with linear responses and saturation of activity after 40 nM (Figure 1A).

Because inner centromere Kin-I stimulator (ICIS) was shown to regulate MCAK microtubule depolymerase activity in vitro, we tested whether it could similarly regulate Kif2a. Four truncation proteins were made from the full-length *Xenopus* ICIS protein: ICIS A ($ICIS^{1–334}$), B ($ICIS^{334–724}$), C ($ICIS^{724–992}$) and D ($ICIS^{992–1338}$) (Figure 1B). The C fragment contains the protein's F box, and the D fragment is the protein's coiled-coil domain. All proteins were expressed in *E. coli* as 6-His fusion proteins and purified on Ni^{2+} columns. An amount of 50 nM Kif2a depolymerized most microtubules by 7 min (Figures 1C and Figure S2). Surprisingly, none of the ICIS fragments could stimulate this activity (Figures 1C and S2 and data not shown).

Phosphorylation of Kif2a by Aurora B for 15 min before the addition of microtubules inhibited Kif2a depolymerase activity (Figures 1C and S2). To test its effects on phosphorylated Kif2a, we then added equimolar amounts of ICIS fragments to the reactions after Aurora B phosphorylation, and depolymerase activity was measured. The ICIS D fragment (the C-terminal, coiled-coil region) did not have an effect on Kif2a alone, but it could restore Kif2a activity after it was first inhibited by Aurora B phosphorylation (Figure 1C). The other fragments had no detectable effect on Kif2a activity (data not shown). A titration of ICIS D into this reaction demonstrates that Kif2a activity increases with ICIS D concentration, and activity approaches that of unphosphorylated Kif2a at stoichiometric amounts of ICIS D (Figure 1D). It appears that Kif2a cannot be stimulated above the unphosphorylated amount of activity because adding five or ten times stoichiometric amounts of ICIS did not activate Kif2a above its uninhibited activity (Figure S3). These data suggest that ICIS binding restores the activity of Kif2a that has been inhibited by Aurora B phosphorylation.

ICIS could reactivate Kif2a by removing the inhibitory phosphorylation or by direct stimulation. Aurora B inhibits MCAK activity by phosphorylation on serine 196 in the neck region of the protein. We found an equivalent site in the neck region of Kif2a, serine 132, and tested whether this site was phosphorylated by Aurora B (Figure S4A). Unfortunately, recombinant Kif2a-S132A mutant protein has no depolymerase activity, making it impossible to conclusively show that this is the key inhibitory site on Kif2a. We made a polyclonal antibody against phospho-S132 of Kif2a. Antibody recognition of Kif2a in vitro required prior phosphorylation by Aurora kinase, demonstrating that this antibody is phosphospecific (Figures 1E and S4B). Moreover, the antibody recognizes a protein at

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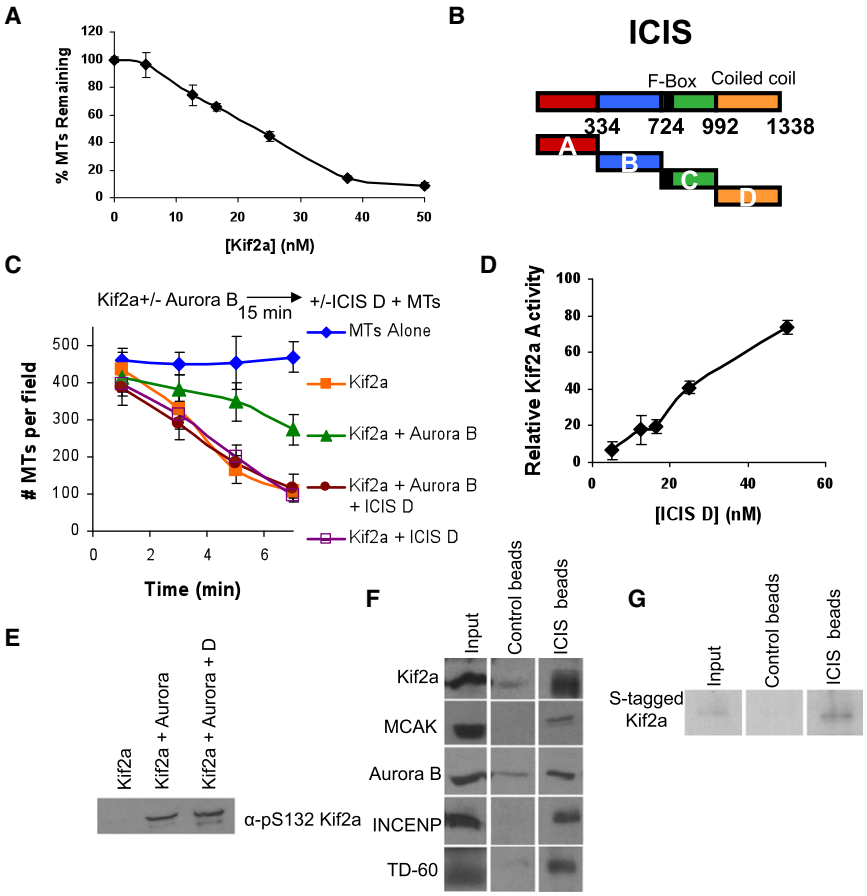

Figure 1. ICIS Reverses Aurora Inhibition of Kif2a In Vitro

(A) Titration of Kif2a into a visual MT depolymerase assay demonstrates depolymerase activity is concentration dependent at 3 min. Error bars represent SD.

(B) Map of *Xenopus* ICIS showing the different truncation proteins that were cloned and bacterially expressed.

(C) ICIS D reactivates Kif2a that has been inhibited by Aurora kinase. Schematic of the in vitro depolymerase assay and quantification of the mean number of microtubules per field in the presence of recombinant Kif2a, Aurora B, and the C-terminal fragment of ICIS (ICIS D). Error bars represent SD.

(D) Depolymerase assays titrating the amount of ICIS D added after prephosphorylation of Kif2a with Aurora B. Relative Kif2a activity was calculated with Kif2a alone as 100% activity and Kif2a + Aurora B as 0% activity. Error bars represent SD.

(E) ICIS does not reactivate by dephosphorylating Kif2a. Immunoblot of α-pS132 Kif2a in an in vitro depolymerase reaction as in (C).

(F) Immunoblots showing that ICIS D interacts with Kif2a, MCAK, Aurora B, INCENP, and TD-60 in *Xenopus* mitotic extracts. ICIS D or 6His-GST was covalently attached to beads and incubated in mitotic extract, and the washed beads were probed for the indicated proteins.

(G) ICIS D interacts directly with Kif2a in vitro. Recombinant S-tagged-Kif2a was incubated with the beads used in (F), and bound Kif2a was detected with S-protein-HRP. Input represents 20% of the reaction.

~85 kD in *Xenopus* M phase extracts containing phosphatase inhibitors, which is the expected size of *Xenopus* Kif2a. To determine whether ICIS is dephosphorylating Kif2a to reactivate activity, we immunoblotted the recombinant microtubule depolymerase reaction with the pS132 antibody (Figure 1E). Phosphorylation of the presumptive inhibitory site did not decrease when ICIS stimulates activity. These data argue that ICIS D activates phosphorylated Kif2a not by dephosphorylation but by direct binding.

To characterize the interactions of ICIS D in vivo, we covalently linked recombinant ICIS D to Sepharose beads. These beads were incubated in mitotic *Xenopus* extracts, and associated proteins were probed by immunoblot. As expected from biochemical experiments, ICIS D binds MCAK and Kif2a (Figure 1F). In addition, ICIS D bound Aurora B and its activators INCENP and TD-60. ICIS D beads were also incubated with purified recombinant Kif2a, which showed that ICIS D and Kif2a interact directly in vitro (Figure 1G).

These data argue for dual regulation of kinesin-13 microtubule depolymerases. First, Aurora B kinase phosphorylates the neck domain of these proteins to inhibit depolymerase activity. Second, a coiled-coil domain on ICIS then reactivates inhibited Kif2a. It is likely that MCAK is regulated in the same way, even though ICIS has been demonstrated to stimulate intrinsic MCAK depolymerase activity [7]. We contend that the purified MCAK used in the original experiments contained both inhibited and uninhibited protein. MCAK purified from baculovirus (which was used in the original experiments) is phosphorylated on S196 and, thus, is partially inhibited (Figure S5). MCAK from baculovirus is not fully phosphorylated, given that we could more than double the pS196 staining after incubation with Aurora B kinase (data not shown). Because we used recombinant Kif2a from bacteria (which is not phosphorylated), it can only be activated by ICIS after Aurora B phosphorylation. However, it is important to test this idea in the future because another important difference between the two experiments is that we used the catalytic portion of Kif2a, whereas previous experiments were performed with full-length MCAK.

Anti-ICIS Injection Results in a Monopolar Spindle Phenotype

To determine whether the C terminus of ICIS regulates kinesin-13 s in vivo, we generated a polyclonal antibody against ICIS D (Figure S6). After affinity purification, this antibody predominantly recognized a protein at the expected molecular weight for ICIS (160 kD). Several smaller bands are also recognized by the antibody, but it only depletes the 160 kD band, suggesting that ICIS is the only protein that is recognized in the native conformation. The antibody against the ICIS D region was added to a microtubule depolymerase reaction, and the antibody blocked the reactivation of Kif2a by ICIS D (Figure 2A).

To determine the importance of the C terminus of ICIS in cultured cells, we injected this antibody into prophase *Xenopus* S3 cells stably expressing GFP-tubulin (Figure 2B and Movie S1). Whereas control IgG-injected cells assemble bipolar spindles by 6–10 min (Figure 2B) and complete anaphase by 45 min, α-ICIS-injected cells (n = 8) form monopolar spindles and maintain a mitotic arrest for at least 70 min

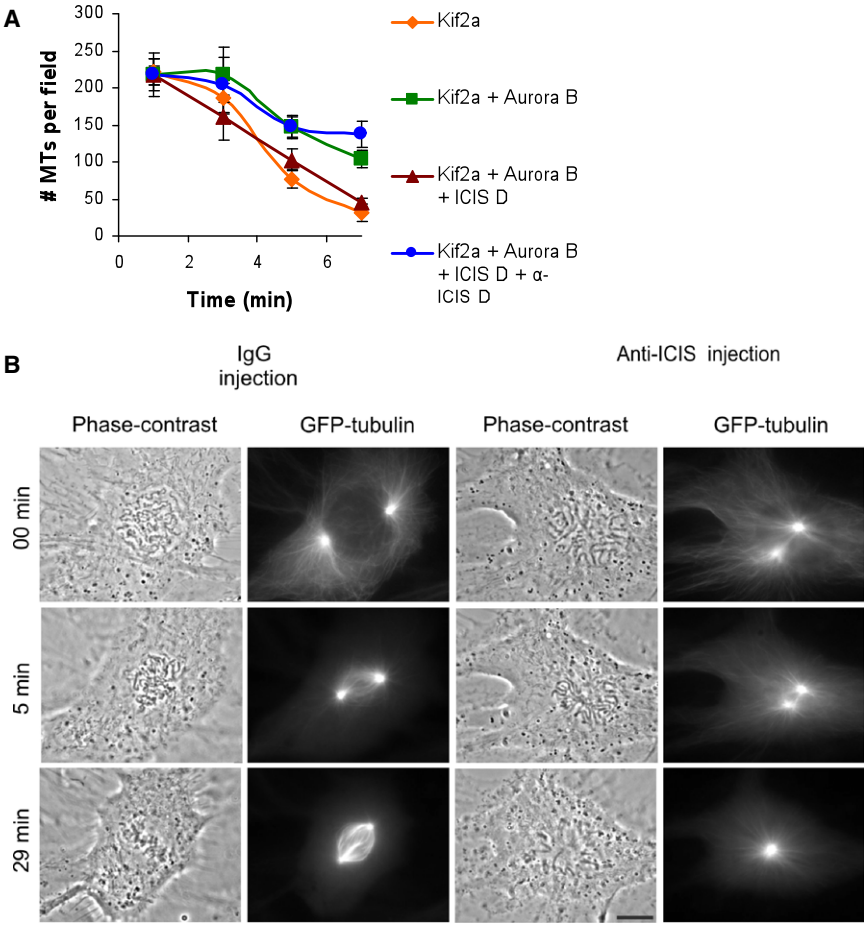

Figure 2. Injection of α-ICIS Antibodies into Cells Gives a Monopolar Spindle Phenotype

(A) ICIS D antibodies inhibit Kif2a reactivation in vitro. Quantification of the mean number of microtubules per field in the presence of recombinant Kif2a, Aurora B, ICIS D, and α-ICIS. Error bars represent SD.

(B) IgG control injected and α-ICIS injected in prophase *Xenopus* S3 cells expressing GFP-tubulin. Time lapse images from Movies S1 and S2. 0 min = time of injection. Scale bar represents 10 μm.

(Movie S2). This result phenocopies siRNA knockdown of Kif2a in U2OS cells [8], although there is no evidence to support Kif2a's role in bipolarity in *Xenopus* cells. The injected antibodies primarily localize to spindle poles (Figure S7). We note that the requirement for ICIS D may not be universal, given that this monopolar spindle phenotype could not be recapitulated in *Xenopus* extracts (data not shown). Because an antibody that blocks the reactivation activity of ICIS also generates spindle defects, these data argue that ICIS reactivation of kinesin-13 s is required for proper spindle function.

Monopolar Spindles Generated by α-ICIS Injection Are Rescued By Coinjecting α-Nuf2

Monopolar spindles can be generated by defects in centrosome separation, regulators of microtubule dynamics (Kif2a and Aurora A), or inhibition of kinesins that crosslink pole-to-pole microtubules (Eg-5 and KSP-1). In the α-ICIS-injected cells, we see separation of poles before nuclear envelope breakdown, arguing for proper centrosome segregation. Previous experiments have shown that inhibition of a class of proteins that regulate K fiber dynamics can be rescued by coinhibition of kinetochore-microtubule binding, whereas monopolarity generated by Eg-5 inhibition is not rescued by this treatment [8]. To classify the monopolarity generated by anti-ICIS injection, we coinjected α-Nuf2 antibodies along with α-ICIS D antibodies into GFP-tubulin-expressing S3 cells (n = 6) (Figure 3 and Movie S3). We found that coinjection did indeed rescue spindle bipolarity, and the cells proceeded through mitosis with a "cut" phenotype, which is expected in a cell with defective kinetochores. These data suggest that knocking out the kinesin-13 reactivating function of ICIS in *Xenopus* cells may generate monopolarity through a similar pathway as siRNA knockdown of Kif2a in U2OS cells.

Kif2a Is Localized to Centromeres in Xenopus Cells

ICIS and MCAK are localized to both centromeres and spindle poles in *Xenopus* [7, 9, 10]. In human U2OS cells, Kif2a localizes to spindle poles during mitosis and Kif2b localizes to centromeres [11]. *Xenopus* appears to only have a gene for Kif2a. We generated a polyclonal antibody against Kif2a to determine its localization in *Xenopus* cells. The Kif2a antibody recognized a single band around 85 kD, the expected size in *Xenopus* (Figure S8A). When the antibody was used for immunofluorescence in *Xenopus* cells, it labeled spindle poles throughout mitosis (Figure S8B, arrows). Kif2a is also localized to most centromeres during prometaphase and to a smaller number of centromeres at metaphase (Figure S8B, inset). Thus, Kif2a, MCAK, and ICIS concentrate at the same cellular locations in *Xenopus* cells.

ICIS Interacts with Aurora B Kinase, Its Substrates, and Its Activators

ICIS binds microtubules and coimmunoprecipitates with members of the chromosomal passenger complex (CPC) and MCAK [7]. We mapped these interactions by covalently attaching the four ICIS fragments to Sepharose beads, incubating them in a *Xenopus* CSF-arrested extract and probing the interacting proteins by immunoblot (Figure 4A). We found that the N terminus of ICIS (ICIS A) interacts with Aurora B and with its activators INCENP and TD-60 [12]. A middle region (ICIS B fragment) interacts with both Kif2a and MCAK (Figure 4A). These data show that ICIS interacts with Aurora B kinase, its activators, and two substrates, Kif2a and MCAK.

To determine which domain of ICIS is responsible for microtubule binding, we added taxol to *Xenopus* M phase extracts and pelleted microtubules and their interacting proteins through sucrose cushions. Microtubules pelleted ICIS B and ICIS C (Figure 4B). ICIS C also bound purified taxol-stabilized microtubules; however, ICIS B did not, and there was a weak interaction with ICIS D. (Figure 4B′).

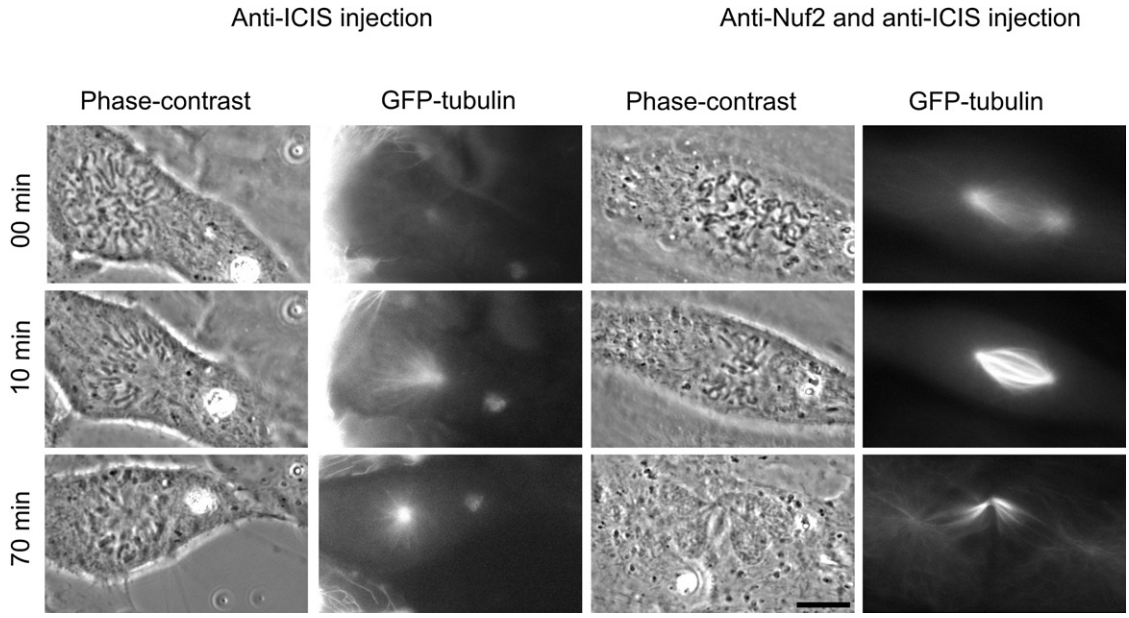

Figure 3. α-ICIS Injection Phenotype Can Be Rescued by Coinjection with α-Nuf2

S3 cells expressing GFP-tubulin were coinjected with α-ICIS and α-Nuf2 antibodies and filmed, and spindle morphology was observed. The coinjected cells had a restored bipolar spindle phenotype. Images are stills from Movie S3. Scale bar represents 10 μm.

We visualized complexes of ICIS C and purified rhodamine-labeled microtubules by fluorescence microscopy. ICIS C bundled microtubules (Figure 4C). The bundles appear to be aggregations of laterally associated microtubules, and the bundles are longer than the individual microtubules used in the assay. The other fragments of ICIS did not bundle microtubules.

Previous to this study, it was not understood why microtubule depolymerases would be phosphorylated on inhibitory sites in cellular locations where their activity is required [1, 3, 6]. This work reveals a novel form of regulation of these kinesins, whereby phosphorylated and inhibited depolymerases can be reactivated by ICIS without being dephosphorylated (Figure 4D). It remains to be seen whether phosphatase activity also plays a role in the reactivation of kinesin-13 family members. Our structure function analysis of ICIS implicates it as a potential scaffold, which could bring together the activator and the inhibitors of kinesin-13 depolymerases (Figure 4D) to facilitate tight spatial and temporal regulation of depolymerase activity in the mitotic spindle.

Experimental Procedures

Protein Expression and Antibody Production

The full-length *Xenopus laevis* ICIS cDNA clone was obtained from R. Ohi (Vanderbilt University, Nashville, TN). A series of bacterial expression constructs were generated by PCR and subcloned into pET30 with 5′ Nco I and 3′ Not I overhangs. The following primers were used to clone the different fragments of ICIS into pET30: for ICIS A ($ICIS^{1–334}$), 5′-CGCGGATC CATGGGATGAGTGTGCAAACAGCAACT-3′ and 5′-TCGAGTGCGGCCGCC TACACTTCTAGCATCACTTCACT-3′; for ICIS B ($ICIS^{334–724}$), 5′-CGCGGATC CATGGGGGTGGATGCTCGTAGGGTGATG-3′ and 5′-TCGAGTGCGGCCGC CTAATCTGCAGATACAGTGCCTTC-3′; for ICIS C ($ICIS^{724–992}$), 5′-CGCGGA TCCATGGGGGATATGGATATCTTGGCCAAC-3′ and 5′-TCGAGTGCGGCC GCCTAGTTACTGCTGCTGAGGAGAT-3′; and for ICIS D ($ICIS^{992–1338}$), 5′-CG CGGATCCATGGGGAACCAAAGGTTCGAGGCACTG-3′ and 5′-TCGAGTGC GGCCGCTCATCTGGGAGACACCGTCGG-3′.

GST-tagged ICIS fragments were cloned by digesting pET30 constructs with NcoI/NotI and replacing the inserts into pGEX-4TS3.

ICIS fragments were expressed in the *E. coli* strain BL21 (DE3 pLysS, Novagen). All 6His-tagged proteins were purified on Ni^{2+}-NTA agarose as instructed by the manufacturer (QIAGEN, Chatsworth, CA). Glutathione S-transferase (GST)-tagged proteins were purified on glutathione agarose beads. Purified proteins were covalently coupled to CNBr-activated Sepharose beads according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Antibodies were produced from the 6His-ICIS D recombinant protein by Covance Research Products (Denver, PA). Antibodies raised against 6His-tagged proteins were purified over corresponding GST-tagged protein columns and vice versa. Affinity-purified antibodies were dialyzed into PBS.

The cDNA clone of human Kif2a (BC033842) was purchased from Open Biosystems (Huntsville, AL). Kif2a aa 530–679 was cloned into pET30a by using primers with the sequences 5′-GCGGATCCCGGATGCCAACTGCTG CTGGTG-3′ and 5′-GATCGGAATTCGTTAAAGGGCACGGGGTCTCTT-3′ to generate the construct Pet30a-Kif2a (530–679). Recombinant protein was expressed in (pLysS) BL21 bacteria for 4 hr at room temperature. This 6His-tagged protein was purified with conventional nickel bead affinity (Qiagen) according to the manufacturer's instructions, and the protein was used to make a polyclonal rabbit antibody (Covance, Denver, PA).

Kif2a minimal domain (aa 118–530) was cloned into pET30a as well by using primers with the sequences 5′-GCGGATCCCGGATGGCAAAAAAGG AATTTGG and 5′-GATCGCGGCCGCTTATGGATCTACAGTCAATTCTTT-3′ and was expressed and purified as above. The protein was dialyzed into supplemented kinase buffer (20 mM tris [pH 7.5], 1 mM MgCl2, 25 mM KCl, 50 mM NaCl, 1 mM DTT, and 5% glycerol).

The pS132 antibody was raised against the peptide C-GPPSRRKpSN (synthesized in Yale's W.M. Keck Protein Core facility, New Haven, CT). The peptide was attached to KLH with SMCC (Pierce), and the resulting conjugate was injected into rabbits to produce polyclonal serum (Covance). For affinity purification, both phospho and nonphospho columns were generated with Sulfo-beads (Pierce) according to the manufacturer's instructions. Antibodies were affinity purified first by counterselection on the nonphosphopeptide before being affinity purified on the phosphopeptide column [13]. Immunoblots were performed as previously described [14], with the exception that, with phosphopeptide antibodies, the PVDF membrane was blocked in 3% BSA.

Visual Microtubule Depolymerase Assays

Microtubule depolymerase assays for Kif2a were performed as described for MCAK [1] with 50 nM Kif2a minimal domain, 50 nM ICIS fragments (unless otherwise noted), and 1 nM Aurora B/INCENP in the assays. In brief, the proteins were incubated with microtubules in CSF-XB (10 mM K-HEPES

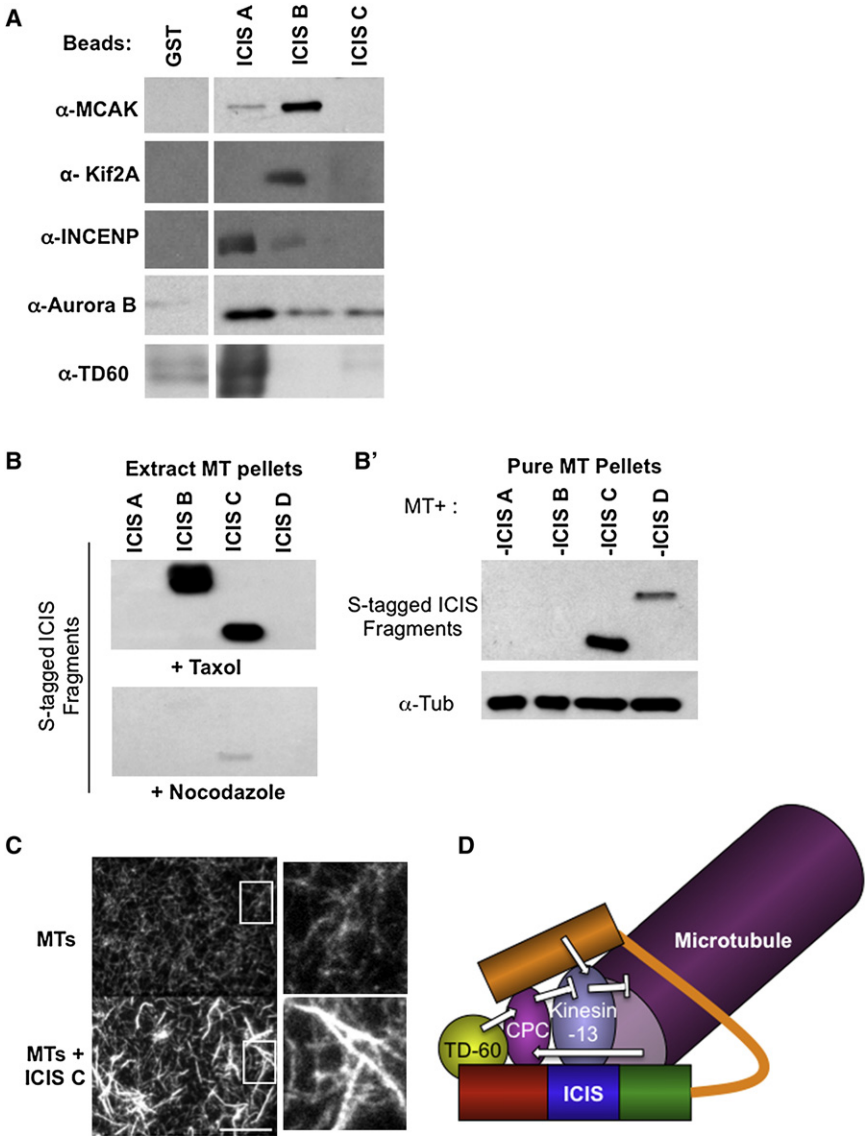

Figure 4. Mapping Where Proteins Bind ICIS

(A) Mapping domains of ICIS interactions. The specified recombinant fragments of ICIS were purified from *E. coli*, covalently attached to beads, and incubated in a mitotic extract. Bound proteins were eluted, and immunoblots were performed on eluates.

(B) Microtubule pelleting assays were performed by adding ICIS fragments and taxol to mitotic extracts, pelleting microtubules, and detecting ICIS by S-protein-HRP (B) or by measuring the affinity of ICIS fragments to purified taxol-stabilized microtubules (B′).

(C) ICIS C bundles microtubules in vitro. Rhodamine-labeled microtubules were incubated with ICIS C and visualized by fluorescent microscopy. Fields taken with a 100× objective are shown, and magnified views are shown to the right. Scale bar represents 10 μm.

(D) Model showing ICIS as a potential scaffold that brings together Kinesin-13 s with its activator (ICIS) and its inhibitor CPC. ICIS also brings Aurora B into contact with its activators TD-60 and microtubules.

[pH 7.7], 100 mM KCl, 2 mM MgCl2, 0.1 CaCl2, 50 mM sucrose, and 5 mM EGTA), with 2 mM ATP, 1 mM DTT, and 15 mM taxol. At the time points indicated, 1 ul samples were taken, diluted into 5 ul fix solution, and spotted onto a slide. Four fields were taken per time point with a 100× objective. The images were acquired as above and analyzed with Metavue software.

Microtubule Pelleting Assays

For microtubule pelleting in CSF extracts, we treated fresh extracts with either 10 μM Taxol or 10 μg/ml Nocodazole (negative control) for 30 min at room temperature. Microtubules and associated proteins were spun through 1 M sucrose cushion. For microtubule pelleting in vitro, we incubated preformed taxol-stabilized pure microtubules (Cytoskeleton, Denver, CO) with different ICIS mutants at room temperature for 30 min. The reaction mixtures were pelleted through 1 M sucrose cushion, washed, and detected by S-protein-HRP or α-tubulin antibody.

Cell Injections

Xenopus laevis S3 cells stably transfected with α-tubulin-GFP were used for microinjections. Cells were kept at 23°C in 70% Leibovitz's L-15 medium containing 15% FBS, L-Glutamine, and penicillin-streptomycin. For microscopy, cells were grown on glass coverslips. Cells were injected with the following antibodies diluted in PBS: rabbit polyclonal anti-ICIS C terminus at a needle concentration of 9 mg/ml and anti-xNuf2 at a needle concentration of 4 mg/ml. Anti-xNuf2 antibodies were characterized previously [15]. Control cells were injected with nonspecific rabbit IgG at a needle concentration of 4 mg/ml. All injections were done before nuclear envelope breakdown (NEB). The cells were analyzed with a Zeiss Axiovert 200 M microscope equipped with Planapochromat 63× (N.A. 1.4) and 100× (N.A. 1.4) objectives and a Hamamatsu Orca ER CCD camera (Hamamatsu Photonics). Images were captured with Metamorph software (Molecular Devices).

Supplemental Data

Supplemental Data include eight figures and three movies and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)00821-5.

Acknowledgments

We thank C. Walczak for the generous gift of the MCAK antibody and the recombinant MCAK protein and R. Ohi for the ICIS cDNA clone. We also thank members of the Burke and Foltz labs and S. Bekiranov for helpful comments. This work was supported by NIH grants 5R01GM50412 to G.J.G. and RO1GM063045 to P.T.S. A.L.K. was supported by Training Grant # T32 GM08136 from NIGMS for Cell and Molecular Biology at the University of Virginia.

Received: August 12, 2008
Revised: March 2, 2009
Accepted: March 3, 2009
Published online: March 26, 2009

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